JOURNAL OF BACTERIOLOGY, Aug. 1968, p. 447-456

Vol. 96, No. 2
Printed in U.S.A.

Mutants of *Aerobacter aerogenes* Capable of Utilizing Xylitol as a Novel Carbon[1]

T. T. WU,[2] E. C. C. LIN,[3] AND S. TANAKA[4]

Department of Biological Chemistry, and Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts 02115

Received for publication 31 May 1968

Wild-type *Aerobacter aerogenes* 1033 is unable to utilize xylitol. A succession of mutants was isolated capable of growth on this compound (0.2%) at progressively faster rates. Whereas the ability to utilize xylitol was achieved in the first-stage mutant (X1) by constitutive production of ribitol dehydrogenase (for which xylitol is a substrate but not an inducer), the basis for enhanced utilization of xylitol in the second-stage mutant (X2) was an alteration of ribitol dehydrogenase. This enzyme was purified from the various mutants. The apparent K_m for xylitol was 0.12 M with X2 enzyme and 0.29 M with X1 enzyme. The X2 enzyme was also less heat stable and, at 0.05 M substrate concentration, had a higher ratio of activity with xylitol compared to ribitol than did the X1 enzyme. The third mutant (X3), with an even faster growth rate on xylitol, produced a ribitol dehydrogenase indistinguishable physically or kinetically from that of X2. However, X3 produced constitutively an active transport system which accepts xylitol. The usual function of this system is apparently for the transport of D-arabitol since the latter is not only a substrate but also an inducer of the transport system in parental strains of X3. The sequence of mutations described herein illustrates how genes belonging to different metabolic systems can be mobilized to serve a new biochemical pathway.

In both *Aerobacter aerogenes* PRL R3 (or NRRL 199; reference 2) and *A. aerogenes* 1033 (reference 32) a single mutation is sufficient to permit their growth on xylitol. This genetic change involves the derepression of ribitol dehydrogenase which can metabolize xylitol but cannot be induced by it (25, 36). Xylitol is thereby converted to D-xylulose, an intermediate in the dissimilation of D-arabitol (28). Thus the utilization of the new carbon source requires the participation of enzymes in two preexistent pathways: one for ribitol and the other for D-arabitol. The scheme in Fig. 1 shows the enzymatic steps in the utilization of the three pentitols by *A. aerogenes* as established in studies at several laboratories (4, 6, 7, 9-11, 19, 35, 37-39, 52).

[1] Part of this work was presented before the American Society of Biological Chemists, Atlantic City, N.J., 1966 (Federation Proc. **25**:338, 1966).

[2] Present address: Department of Biomathematics, Cornell University Graduate School of Medical Sciences, New York, N.Y. 10021.

[3] Supported by a Research Career Development Award from the Public Health Service.

[4] Present address: Department of Nutritional Pathology, Tokushima University Medical School, Tokushima, Japan.

Mutant X1, isolated in this laboratory from *A. aerogenes* 1033, is capable of doubling every 4 hr at 37 C in a medium containing 0.2% xylitol. It produces ribitol dehydrogenase constitutively. A derivative of this mutant, X2, with a doubling time of about 2 hr was obtained by repeated growth on xylitol under the same conditions as those for X1. Strain X2 produces an altered ribitol dehydrogenase with enhanced activity toward xylitol (25). A tertiary mutant, strain X3, with a doubling time of 55 min has now been isolated by further growth cycling on xylitol at a reduced concentration (0.05%). The present study concerns primarily the nature of the third-stage mutant.

MATERIALS AND METHODS

Bacteria. The mutants studied were derived from strain 5P14, an arginine and guanine auxotroph of *A. aerogenes* 1033, provided by Boris Magasanik. The double nutritional requirement was used to distinguish mutant progeny from possible contaminating organisms. Procedures for the isolation of strain X1 from 5P14 and strain X2 from strain X1 have been reported (25). Strain X3 was selected from strain X2 for faster growth on xylitol by recycling cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1) in a medium containing 0.05% xylitol as the source of carbon

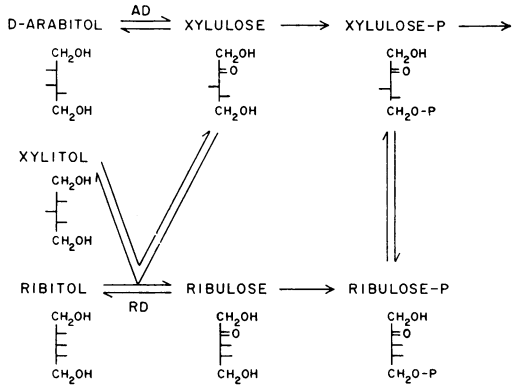

FIG. 1. *Metabolic pathways for* D-*arabitol, ribitol, and xylitol. AD represents* D-*arabitol dehydrogenase and RD, ribitol dehydrogenase.*

and energy. Strain A^c was isolated from strain 5P14 for constitutive utilization of D-arabitol (29).

Chemicals. ^{14}C-D-arabitol (1.5 c per mole) was purchased from New England Nuclear Corp., Boston, Mass.; D-mannitol-*1*-^{14}*C* (25 c per mole) from Nuclear-Chicago Corp., Des Plaines, Ill.; ^{14}C-xylitol (3.8 c per mole) from Nuclear Research Chemicals, Inc., Orlando, Fla.; D-mannitol from Eastman Kodak Co., Rochester, N.Y.; D-arabitol, ribitol, xylitol, and D-sorbitol from Pfanstiehl Laboratories, Waukegan, Ill.; sodium succinate from Mallinckrodt Chemical Works, St. Louis, Mo.; casein acid hydrolysate from Nutritional Biochemicals Corp., Cleveland, Ohio; nicotinamide adenine dinucleotide (NAD) from Pabst Laboratories, Inc., Milwaukee, Wis.; sodium phosphoenolpyruvate (PEP) from Calbiochem, Los Angeles, Calif.; and ammonium sulfate (enzyme grade) from Mann Research Laboratories, Inc., New York, N.Y.

Growth of cells. The basal medium contained 0.034 M NaH_2PO_4, 0.064 M K_2HPO_4, 0.02 M $(NH_4)_2SO_4$, 3×10^{-4} M $MgSO_4$, and 1×10^{-6} M each of $FeSO_4$, $ZnCl_2$, and $CaCl_2$. The solution was titrated with HCl to a final *p*H of 7.0. Unless otherwise specified, the indicated sources of carbon and energy were added in the following final concentrations: polyol, 0.2%; succinate, 0.4%; and casein hydrolysate, 1%.

For measurement of growth rates, cells were inoculated at low density into 15 ml of media and incubated at 37 C in 300-ml Erlenmeyer flasks with side arm and swirled at about 240 cycles per min on a rotary shaker. Growth was monitored turbidimetrically in a Klett colorimeter with a 420-nm filter. Doubling times were determined during early periods of growth before significant depletion of the carbon source took place. Cells for the assay or purification of enzymes were grown in 1 liter of medium in 2-liter Erlenmeyer flasks. The procedure for cell disruption has been described (48).

Enzyme assays. Dehydrogenase activities were carried out at 25 C by folllowing the appearance of reduced NAD at 340 nm in a Gilford model 2000 spectrophotometer. The assay mixture (in a final volume of 3.0 ml) contained 0.67 mM NAD, 100 mM sodium carbonate at *p*H 10.9, 50 mM substrate, and enzyme extract. Protein was measured with the biuret reagent (14). A unit of activity represents 1 μmole of reduced NAD formed per min. Tests for phosphorylation of D-mannitol and D-arabitol by the PEP-dependent system (23) were carried out as previously described (48), except that the assay mixtures were not fortified with enzyme I or the heat-stable protein (HPr).

Purification of ribitol dehydrogenase. Unless otherwise specified, all procedures were carried out at 0 to 5 C. Cells from 6 liters of culture, grown to the stationary phase, were extracted as previously described (48). About 80 ml of streptomycin sulfate (20%) was slowly added to the extract to a final concentration of 2%. The mixture was stirred for about 20 min, and the precipitate was removed by centrifugation. Saturated ammonium sulfate was added to the supernatant fraction with constant stirring to 40% saturation. After 20 min of equilibration, the precipitate was removed by centrifugation. The ammonium sulfate concentration in the supernatant fraction was raised to 50% saturation. The precipitate was collected by centrifugation and dissolved in 70 ml of 0.01 M sodium phosphate at *p*H 7.0. The solution was dialyzed against 2 liters of the same buffer for 3 hr after which the contents were mixed with 5 g of diethylaminoethyl (DEAE)-cellulose for 20 min. The cellulose with the adsorbed enzyme was separated by filtration and suspended in 3.5 ml of 0.2 M sodium phosphate at *p*H 7.0. The supernatant fraction containing the eluted enzyme was then diluted 20-fold with water and layered on a column (2.54 $\times$ 51 cm) of DEAE-cellulose. Protein was eluted by 1 liter of sodium phosphate, *p*H 7.0, in a linear gradient from 0.01 to 0.2 M. Five to seven of the most active fractions (10 ml each) were pooled and precipitated by addition of solid ammonium sulfate to 80% saturation. The precipitate was dissolved in 2 to 3 ml of 0.01 M sodium phosphate at *p*H 7.0 and layered on a column (2.54 $\times$ 25 cm) of Sephadex G-200 equilibrated with the same buffer. About 160 ml of the same phosphate buffer was then passed through the column, and 4-ml fractions were collected. The major peak of material absorbing at 280 nm coincided with the peak enzyme activity. Ten fractions containing about 40% of the total enzyme activity were pooled; the protein was precipitated by ammonium sulfate (80% saturation) and dissolved in 1.5 ml of 0.01 M sodium phosphate, *p*H 7.0.

Ultracentrifugal analysis of enzyme preparations. The purified enzymes were dissolved in 0.01 M sodium phosphate, *p*H 7.0, at a protein concentration of 10 mg/ml and centrifuged at 20 C in a model E Spinco analytical centrifuge at 59,780 rev/min.

Disc gel electrophoresis. Acrylamide gel electrophoresis was performed with the use of the Buchler apparatus (Buchler Instruments, Inc., New York, N.Y.) following the method of Ornstein and Davis (41). The gel columns, consisting of 6.5% acrylamide and 0.17% *N,N'*-methylene-bisacrylamide, were 0.9 $\times$ 17 cm. Current was maintained at about 5 ma per tube. After about 6 hr, the gel was fixed in 47% methanol and 6% acetic acid for 5 hr and stained in 0.1%

buffalo black NBR and 7% acetic acid for 1 hr before rinsing with water.

Purification of ^{14}C-xylitol. Since cells of strain A^c (constitutive for D-arabitol utilization) grown on succinate were incapable of utilizing xylitol, they were used to scavenge from the shipment of labeled xylitol trace contaminants which could interfere with the transport experiments. The cells were suspended at a density of 2×10^7 per ml in basal inorganic medium containing 10^{-4} M ^{14}C-xylitol and incubated for 2 hr at 37 C, after which the medium was recovered by filtration through a Millipore disc (0.45-μ pore size). By this procedure the amount of impurity removed was about 2% of the total radioactivity in the sample.

Uptake of labeled pentitol. Assays of ^{14}C-pentitol uptake by cells were carried out at 25 C in basal salt medium containing 5×10^{-5} M ^{14}C-substrate and 40 μg chloramphenicol per ml. The cells were harvested by centrifugation when the exponentially growing culture reached a density of 100 Klett units and suspended at a concentration of 600 μg (wet weight) per ml of assay mixture. The rate of uptake remained nearly linear for 1 hr in the case of xylitol but only for about 2 min in the case of D-arabitol. The exposure was terminated by abrupt removal of the medium on a Millipore filter (15). After drying, radioactivity in the cells was measured in an end-window gas-flow counter.

Paper chromatography. Labeled compounds recovered from cells were concentrated in a rotary evaporator under vacuum and separately spotted on Whatman no. 1 paper. Descending chromatography was carried out at 0 to 5 C with a solvent system consisting of 95% ethyl alcohol and 5% water. When the solvent front had migrated 5.1 cm, the paper was dried and cut into strips perpendicular to the solvent front. The pentitols were located chemically by color development (13). Each strip was then cut into 1.3-cm pieces which were glued to planchets with rubber cement and counted for radioactivity in an end-window gas-flow counter.

RESULTS

Ribitol dehydrogenase of strains X1, X2, and X3. Although strain X3 is the mutant growing fastest on xylitol, its doubling time (55 min) on the novel carbon source was still significantly longer than the doubling time on D-arabitol (45 min). So long as the doubling time on xylitol exceeded that on D-arabitol, the production of D-xylulose must be rate-limiting for growth, since this ketopentose is the obligatory intermediate that is common to the newly established xylitol pathway and the preexisting D-arabitol pathway. The pivotal role of D-xylulose was demonstrated by the facts that a mutant lacking D-arabitol dehydrogenase failed to utilize D-arabitol (48) and that a derivative of strain X1 which no longer could produce ribitol dehydrogenase concomitantly lost its ability to grow both on xylitol and on ribitol (25).

Ribitol dehydrogenase, responsible for the conversion of xylitol to D-xylulose, was found to be produced constitutively in all three mutant strains. The specific activity of this enzyme in crude extracts depended upon the phase of growth as well as the carbon source. In general, extracts of strain X1 harvested during exponential growth on xylitol exhibited specific activities (measured with 0.05 M xylitol) in the neighborhood of 0.1 unit, whereas extracts of strains X2 and X3 harvested from similar growth conditions had about 1.5 to 2 times that value.

The enzyme was purified from each strain to detect possible stepwise modification. Table 1 summarizes the purification procedure and enzyme yields observed with a preparation from strain X1. The same procedure was used for the purification of the enzymes from strains X2 and X3. After the last stage of purification, the major component in each of the three samples migrated at the same rate during ultracentrifugation, as typified by the pattern in Fig. 2. The asymmetry of the boundary indicates that the preparations were not yet homogeneous. The electrophoretic patterns of these preparations are shown with those of a crude extract in Fig. 3.

The enzymes from strains X2 and X3 were partially inactivated after passage through Sephadex. To improve the yield of enzyme activity for the studies described below, the Sephadex treatment was replaced by a second ammonium sulfate fractional precipitation. Saturated ammonium sulfate was added dropwise to the enzyme sample until the solution became turbid. After equilibration for about 20 min, the precipitate was collected by centrifugation. The supernatant fraction was enriched further with ammonium sulfate until the solution again became turbid. A series of five fractions were thus collected. The three fractions containing the highest specific activity of the

TABLE 1. *Purification of ribitol dehydrogenase from strain X1*[a]

Step	Protein (mg)	Specific activty (units/mg of protein)	Yield (%)
Crude extract	2,500	3.3	100
Streptomycin supernatant solution	—	—	96
Ammonium sulfate precipitation	730	6.5	57
Dialysis and DEAE-cellulose adsorption	620	7.4	55
DEAE-cellulose column chromatography	30	85	31
Sephadex G200 fractionation	8	123	12

[a] Cells were grown on casein hyrolysate to stationary phase. Ribitol was the substrate for all enzyme assays.

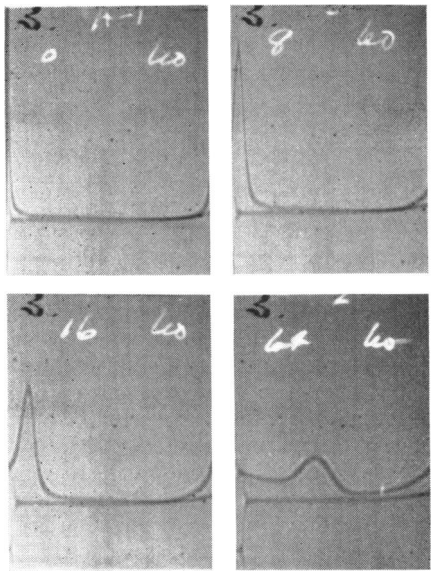

FIG. 2. *Ultracentrifugal analysis of ribitol dehydrogenase of strain X1. Pictures were taken at 0, 8, 16, and 64 min. The boundary moved from left to right.*

enzyme were pooled. The complete procedure for the purification of the enzyme from strains X2 and X3 is given in Table 2.

Data on the stability and some of the catalytic properties of ribitol dehydrogenase from the three mutants are shown in Table 3 and Fig. 4. The wild-type enzyme, prepared from strain X1, displayed an apparent K_m of 3.1 mM for ribitol and 0.29 M for xylitol, thus resembling the ribitol dehydrogenase crystallized from *A. aerogenes* PRL R3 whose corresponding K_m values were 2.6 mM and 0.29 M, respectively (7). The ribitol dehydrogenase from strain X2 was apparently altered by mutation. At 0.05 M substrate, this enzyme displayed a higher ratio of activity with xylitol compared to ribitol than did the enzyme from the parental strain X1. This change in specificity involved both the K_m and the V_{max} values. A modification of the structure of the X2 enzyme was confirmed by its reduced thermal stability. When the enzyme from strain X3 was examined similarly for substrate specificity and temperature sensitivity, it was found to be indistinguishable from the X2 enzyme. Hence, the dissimilar growth rates on xylitol between these two strains are not explicable on the basis of ribitol dehydrogenase. This suggested that either the entry process of xylitol was being affected [i.e., our original assumption that short chain polyols could enter the cell at adequate rates by free diffusion (25) was invalid] or an additional pathway was made available for the dissimilation of the compound.

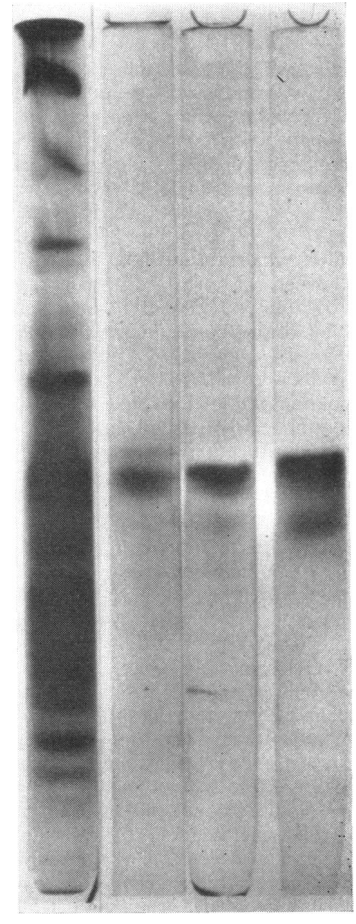

FIG. 3. *Gel electrophoresis of purified ribitol dehydrogenase. The first column on the left represents the electrophoretic pattern of a crude extract of strain X1 (4 mg of protein). The three samples to the right (each containing about 0.1 mg of protein) were purified from strains X1, X2, and X3 in that order.*

Growth rates of mutants as a function of xylitol concentration. To test whether permeability of the cells to xylitol was important in determining their growth characteristics, we measured the doubling times of all three strains in media containing various concentrations of the carbon source. The wild-type strain was included as the control. The reciprocal of the doubling time (growth rate) was plotted against the concentration of xylitol (Fig. 5). Several features deserve notice. (i) The curves for strains X1 and X2 do not reflect typical saturation kinetics but are sigmoid; in contrast, the curve for strain X3 is of the Michaelis-Menten type. (ii) Strain X3 grew readily in 0.05% xylitol (the condition under which this mutant was selected), while strains X1 and X2 grew hardly at all. (iii) The three mutants grew at different rates in 0.2% xylitol, the standard condition for our stud-

TABLE 2. *Purification of ribitol dehydrogenases from strains X2 and X3*[a]

Step	Protein (mg)		Specific activity (units/mg of protein)		Yield (%)	
	X2	X3	X2	X3	X2	X3
Crude extract	2,500	2,600	0.78	1.3	100	100
Streptomycin supernatant solution	—	—	—	—	97	99
Ammonium sulfate I	635	700	1.9	3.6	60	73
Dialysis and DEAE-cellulose adsorption	480	440	2.4	4.4	55	56
DEAE-cellulose column chromatography	19	25	20	24	18	17
Ammonium sulfate II	7.2	10	28	41	10	11

[a] Cells were grown on casein hydrolysate to stationary phase. Ribitol was the substrate for all enzyme assays.

TABLE 3. *Properties of purified ribitol dehydrogenase*

Strain	Activity ratio (xylitol/ribitol)		K_m for xylitol[a]	K_m for ribitol[a]	Half-life[b] at 60 C
	At 0.05 M substrate	At V_{max} (calculated)			
			(M)	(M)	(min)
X1	0.046	0.29	0.29	0.0031	2.8
X2	0.193	0.62	0.12	0.0025	0.7
X3	0.195	0.67	0.13	0.0020	0.7

[a] The values of K_m were determined by the Lineweaver-Burk double reciprocal plot.

[b] Heat inactivations were carried out with samples containing 0.1 mg of protein per ml in 0.01 M sodium phosphate buffer, *p*H 7.0.

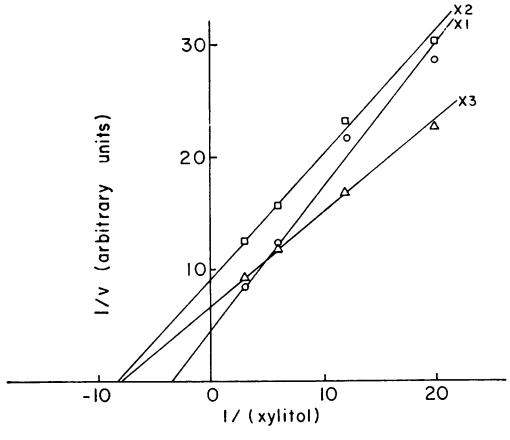

FIG. 4. *Reciprocal plots relating reaction velocity to substrate concentration.*

ies. (iv) The growth rates of all three mutants converged to approach a doubling time of 50 min as the concentration of xylitol in the medium exceeded 0.2%. (v) Wild-type cells (in which ribitol dehydrogenase is inducible) failed to grow at all concentrations of xylitol tested.

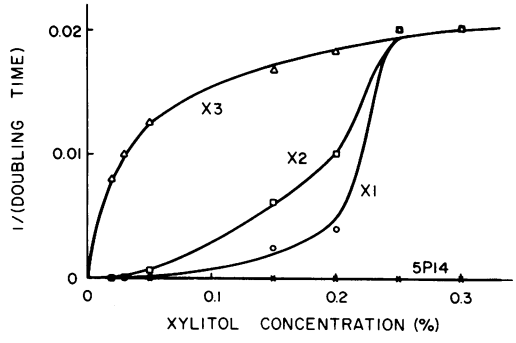

FIG. 5. *The reciprocal of the doubling time (min) of the three mutants and the wild-type strain as a function of xylitol concentration in the medium.*

The most revealing fact is that the concentration of xylitol (approximately 2 mM) affording half maximal growth rate for X3 was two orders of magnitude lower than the apparent K_m of its ribitol dehydrogenase for xylitol (Table 3). [The apparent K_m of the enzyme determined at *p*H 10.9 was found valid also at *p*H 7.5 by the use of an assay dependent upon dye reduction (48).] This discrepancy and the fact that all three mutants could grow equally rapidly on xylitol at high concentrations indicate that an active transport system is derepressed genetically in strain X3.

Uptake of ^{14}C-labeled substrate. Cells of strains X1, X2, and X3, grown out on casein hydrolysate, were tested for their ability to accumulate radioactive label when incubated in the presence of ^{14}C-xylitol. The results (Table 4) showed that such cells from X3 were more active in the uptake of the substrate than were those of the other two strains, supporting the idea that a transport system active on xylitol had become constitutive.

In an attempt to determine the usual function of the putative transport system, the uptake of ^{14}C-xylitol (5×10^{-5} M) was tested in the presence of each of several other straight chain polyols (10^{-4} M) known to be utilized by the

TABLE 4. *Uptake of ^{14}C-pentitols by suspended cells*[a]

Strain	Growth medium	^{14}C-xylitol uptake (counts/15 min)	^{14}C-D-arabitol uptake (counts/2 min)
X1	Casein hydrolysate	60	440
X2	Casein hydrolysate	60	430
X3	Casein hydrolysate	775	6,350
A^c	Casein hydrolysate	770	6,780
X1	D-Arabitol	210	1,720
X2	D-Arabitol	275	1,830
X3	D-Arabitol	760	6,550
A^c	D-Arabitol	1,020	5,330

[a] Data are the values after subtraction of blanks (less than 50 counts/min) in which cells were heated at 60 C for 30 min before exposure to substrate.

progenitor strains of the X mutants: glycerol (30–32, 44); ribitol (19); D-arabitol (28); mannitol (33, 48); and sorbitol (33). Of these only D-arabitol was inhibitory. The possibility that D-arabitol might be the normal substrate for this uptake system was pursued further by testing the ability of the compound to induce a similar mechanism in strains X1 and X2. Cells of these two strains (but not of strain X3) took up more labeled xylitol when grown on D-arabitol instead of casein hydrolysate (Table 4).

When labeled D-arabitol was tested directly as a substrate for uptake by all three strains grown on casein hydrolysate or on D-arabitol, the patterns of accumulation of labeled material were parallel to those observed with xylitol as the substrate but much higher. Thus, it appears that the system studied belongs to the D-arabitol pathway in wild-type cells. This conclusion was corroborated by the fact that the mutant A^c, selected directly from strain 5P14 for constitutive utilization of D-arabitol, behaved similarly to strain X3 in the transport experiments (Table 4).

Identification of free pentitols accumulated by the cells. To determine whether the uptake process involved the concentration of the free substrate against a gradient, it was necessary to carry out the test with cells incapable of further metabolism of the accumulated compound. Strain A^c grown on casein hydrolysate produced the D-arabitol uptake system and D-arabitol dehydrogenase at high levels but negligible amounts of ribitol dehydrogenase. Since D-arabitol dehydrogenase is not active on xylitol, these cells would be expected to accumulate the compound as the free pentitol. A 10-ml cell suspension, at a density of 100 Klett units, was incubated in the presence of ^{14}C-xylitol and chloramphenicol for 30 min. If all the ^{14}C-material accumulated during this period represented the unaltered substrate, the concentration gradient achieved would be about 40-fold. [The cellular water content of *Escherichia coli*, 2.7 μliters per mg of dry weight (51) was used for this estimate.] The cells were then collected on a Millipore filter disc and washed with inorganic basal medium, after which they were resuspended in 10 ml of a salt-free solution of unlabeled xylitol (10^{-2} M) and incubated for another 30 min to permit recovery of the intracellular ^{14}C-xylitol by exchange. More than 75% of the accumulated labeled material was thus released into the incubation solution, which was concentrated to 0.1 ml for chromatographic analysis as described under Methods. The recovered compound was indistinguishable from standard ^{14}C-xylitol; the comparison was made by running a portion of the unknown in parallel with another portion mixed with an equivalent amount (in counts) of labeled authentic compound. The radioactive material in the two samples migrated as a single peak and at the same rate. In each case, this peak coincided with the chemically revealed spot. Similar results were also obtained with an acid and an alkaline solvent system (34).

An analogous experiment was carried out with cells of strain X3 grown on casein hydrolysate employing ^{14}C-D-arabitol as the nonmetabolizable substrate. These cells were found to be genetically derepressed only in the uptake mechanism for D-arabitol and not in its dehydrogenase. Although ribitol dehydrogenase was produced constitutively, this enzyme was inactive on D-arabitol. The radioactive material recovered from such cells was likewise found to be chromatographically indistinguishable from standard ^{14}C-D-arabitol.

Test for a PEP-dependent phosphorylating system for D-arabitol. Certain carbohydrates are not transported against a concentration gradient but are trapped intracellularly by a phosphorylation reaction dependent upon PEP; the products thus formed can be metabolized without prior hydrolysis of the phosphoric ester (8, 16, 22, 45–47, 49). Although the D-arabitol taken up by the cells was recoverable by exchange with external carrier, phosphorylation of the pentitol inside the cell could not be excluded on this basis. For example, it was found that labeled α-methyl glucoside trapped in the phosphorylated form by the action of the PEP-dependent system (22) could be released as the free sugar when the cells were incubated with cold α-methyl glucoside (50). A direct test was, therefore, performed by incubating an extract of wild-type *A. aerogenes* (grown on D-arabitol) with labeled D-arabitol in the presence of PEP. Negligible phosphorylating activity was found. In contrast, under similar conditions an

extract of cells grown on mannitol catalyzed actively the phosphorylation of labeled mannitol (Table 5). Hence, it seems that D-arabitol can be concentrated by the cell without involvement of the PEP system as was concluded to be the case for the transport of lactose by *E. coli* (22). It should be emphasized that in neither of the above two cases is phosphorylation expected to play a role in the capture of the substrate in view of the fact that the first metabolic enzyme attacks the free carbohydrate rather than a phosphorylated derivative.

Induction of the D-arabitol system in cells grown on xylitol. If growth on xylitol is limited by D-xylulose production, the intracellular concentration of this ketopentose should be progressively higher in strains X1, X2, and X3. It will be noted that D-xylulose not only gives rise to further metabolites in the D-arabitol pathway but may also be reversibly converted to D-arabitol itself (Fig. 1). Hence, irrespective of which compound in this series acts as the direct inducer of the D-arabitol system, the degree of induction is expected to be influenced by the level of D-xylulose; i.e., the expression of the genes in the D-arabitol pathway should serve as an internal indicator for the level of the D-xylulose pool. To test the postulate, all three mutants were inoculated at low density in 0.2% xylitol, a concentration giving large differences in growth rate. The cells were harvested in the exponential phase of growth (around 100 Klett units). A portion of each was measured for the uptake of ^{14}C-xylitol (by the D-arabitol carrier), and the remainder was disrupted and assayed for D-arabitol dehydrogenase, a gratuitous enzyme during growth on xylitol. The amount of induction of the D-arabitol system increased sequentially in the mutants as expected (Table 6). These findings, together with the fact that at high xylitol concentrations all mutants grow at the same rate, lead to the conclusion that the stepwise increase in growth rate on the novel carbon source was achieved by using a single pathway more effectively rather than by recruiting auxiliary routes of metabolism.

TABLE 5. *PEP-dependent phosphorylation*

Cells grown on	Test substrate	Relative activity[a]
D-Arabitol.....	^{14}C-D-arabitol	3
D-Mannitol.....	^{14}C-D-mannitol	100[b]

[a] Assay mixtures were not fortified with enzyme I or HPr (23) to avoid possible bias on the activities of the crude extracts with the respective test substrates. It is not certain that only one kind of enzyme I or of HPr exists.

[b] 1.8 nmoles per mg of protein per min at 25 C.

TABLE 6. *Xylitol uptake and D-arabitol dehydrogenase activity in mutant cells grown on xylitol*[a]

Strain	Doubling time (hr)	^{14}C-xylitol uptake (counts/15 min)	D-Arabitol dehydrogenase (units/ mg of protein)
X1	4.1	104	0.01
X2	1.7	185	0.07
X3	0.9	685	0.30

[a] Cells were inoculated at low density into media containing 0.2% xylitol and were harvested at 100 Klett units.

DISCUSSION

With the demonstration of an active transport system for D-arabitol which also acts on xylitol (Fig. 6), the major growth characteristics of the three mutants (Fig. 5) become interpretable. Strain X3 produces this system constitutively, and, therefore, its ability to extract xylitol from the medium should essentially reflect the kinetic characteristics of the transport process. The circumstances are more complex with strains X1 and X2 in which D-arabitol transport system is inducible. The degree of induction of the transport system can be precariously dependent upon the xylitol concentration in the medium because, as this concentration is gradually raised, the rate of entry into the cell (either exclusively through the D-arabitol transport system already present in the membrane or through other channels as well) should increase; and, as the level of intracellular xylitol gets higher, its rate of conversion to D-xylulose would accelerate, which would in turn enhance the induction of the proteins of the D-arabitol pathway. (Note the K_m values of ribitol dehydrogenase for xylitol in Table 3.) The operation of this positive feedback loop would account for the sigmoid nature of the growth curves. The threshold concentration of xylitol in the medium bringing about the sharp change in growth rate is lower for strain X2 than for strain X1, peusrmably because the dehydrogenase in the former strain has a lower K_m for xylitol. In addition, the growth characteristics of these mutant strains in the critical regions of xylitol concentration may be complicated by heterogeneous induction among the cells (3, 40).

A separate transport system for ribitol is represented in Fig. 6. This inducible system was encountered during the experiments on the uptake of pentitols, but its specificity remains to be characterized.

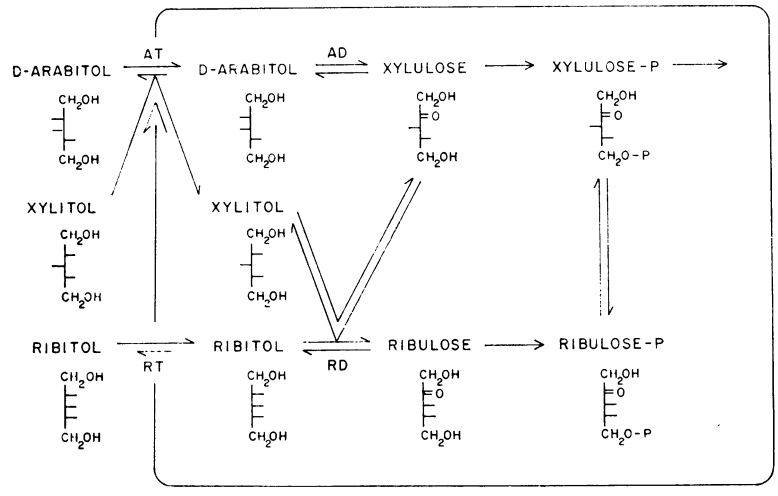

FIG. 6. *The pathways for transport and catabolism of* D-*arabitol, ribitol, and xylitol. AT represents the* D-*arabito transport system and RT, the ribitol transport system.*

There are now several reported cases in which compounds enter bacterial cells through transport systems primarily serving other functions. These include the transport of diaminopimelate by a system active on cystine (24), the transport of certain β-galactosides by the system for melibiose (42), the transport of galactose by systems for lactose, melibiose, and methylgalactoside (12, 43), the transport of raffinose by the system for lactose (26; S. Schaefler, Bacteriol. Proc., p. 54, 1967), the transport of glucose by the system for galactose (46), and, in the present work, the transport of xylitol by the system for D-arabitol. In some cases, it has also been shown that the nonphysiological substrates were also concentrated against a gradient. The unusual substrates, however, are often distinguishable by their relatively poor apparent affinities for the recognition sites. The failure of many transport systems to discriminate against nonphysiological substrates at high concentration may mean that mere binding of the small molecule to a site on the protein is sufficient for passage across the membrane.

The development of a transport mechanism as an eventual step in the evolution of a catabolic sequence had been anticipated (25) on the basis of the retroaddition principle of Horowitz (17). The precise sequence of mutations leading to the establishment of a novel pathway, however, is much less predictable because it should depend not only on the particular genome of the organism but also on the selective conditions.

Gene duplication in tandem (5) followed by gene divergence (27) has been invoked to account for the apperance of multicistronic operons encoding proteins catalyzing a series of metabolic reactions (18). Although a series of proteins with homologous function, such as myoglobin and the various subunits of hemoglobins, may very well have emerged in this manner (20), a metabolic sequence involving dissimilar reaction mechanisms cannot be expected to arise this way. For example, to elaborate a xylitol pathway such as the one considered here would require that the gene for the NAD-linked "xylitol" dehydrogenase be descended from a copy of the gene for the adenosine triphosphate-dependent D-xylulose kinase and that the gene for the "xylitol" transport protein be similarly descended from the gene for the dehydrogenase. It is highly improbable that proteins can be so radically transformed by a few mutations. On the other hand, one sees that the roles of structural genes can be readily widened simply by derepression. Further modifications may then occur with improvement of the efficiency of the new functions. Once the utility of certain cistrons is thus broadened, subsequent gene duplication may be favored by selection. Such a process would allow better adaptation to the new demand without having to sacrifice the old capacities. If the enzymes recruited into a novel pathway are from different preexistent metabolic systems, the corresponding genes are likely to be dispersedly located. However, should coordinate control be advantageous for a given system, these genes could be collected into a single operon by translocation (21). Since the contiguity of genes may be the result of either rearrangement or duplication of deoxyribonucleic acid segments, linkage by itself is not a reliable criterion for determining the stage of gene evolution.

ACKNOWLEDGMENTS

We thank J. P. Alpers, H. Paulus, and H. H. Winkler for helpful discussions.

This investigation was supported by grant GB-5854 from the National Science Foundation and by Public Health Service grant GM-11983 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. Altermatt, H. A., F. J. Simpson, and A. C. Neish. 1955. The anaerobic dissimilation of D-ribose-1-C^{14}, D-xylose-1-C^{14}, D-xylose-2-C^{14}, and D-xylose-5-C^{14} by *Aerobacter aerogenes*. Can. J. Biochem. Physiol. **33**:615–621.
3. Benzer, S. 1953. Induced synthesis of enzymes in bacteria analyzed at the cellular level. Biochim. Biophys. Acta **11**:383–395.
4. Bhuyan, B. K., and F. J. Simpson. 1962. Some properties of the D-xylulokinase of *Aerobacter aerogenes*. Can. J. Microbiol. **8**:737–745.
5. Bridges, C. B. 1936. The bar "gene" a duplication. Science **83**:210–211.
6. Fossitt, D., R. P. Mortlock, R. L. Anderson, and W. A. Wood. 1964. Pathways of L-arabitol and xylitol metabolism in *Aerobacter aerogenes*. J. Biol. Chem. **239**:2110–2115.
7. Fossitt, D. D., and W. A. Wood. 1966. Pentitol dehydrogenases of *Aerobacter aerogenes*, p. 180–186. *In* W. A. Wood (ed.), Methods in enzymology, vol. 9. Academic Press, Inc., New York.
8. Fox, C. F., and G. Wilson. 1968. The role of a phosphoenolpyruvate-dependent kinase system in β-glucoside catabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **59**:988–995.
9. Fromm, H. J. 1958. Ribitol dehydrogenase. I. Purification and properties of the enzyme. J. Biol. Chem. **233**:1049–1052.
10. Fromm, H. J., and J. A. Bietz. 1966. Ribitol dehydrogenase. IV. Purification and crystallization of the enzyme. Arch. Biochem. Biophys. **115**:510–514.
11. Fromm, H. J., and D. R. Nelson. 1962. Ribitol dehydrogenase. III. Kinetic studies with product inhibition. J. Biol. Chem. **237**:215–220.
12. Ganesan, A. K., and B. Rotman. 1966. Transport systems for galactose and galactosides in *Escherichia coli*. I. Genetic determination and regulation of the methylgalactoside permease. J. Mol. Biol. **16**:42–50.
13. Godin, P. 1954. A new spray reagent for paper chromatography of polyols and cetoses. Nature **174**:143.
14. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. **177**:751–766.
15. Hayashi, S., J. P. Koch, and E. C. C. Lin. 1964. Active transport of L-α-glycerophosphate in *Escherichia coli*. J. Biol. Chem. **239**:3098–3105.
16. Hengstenberg, W., J. B. Egan, and M. L. Morse. 1967. Carbohydrate transport in *Staphylococcus aureus*, V. The accumulation of phosphorylated carbohydrate derivatives, and evidence for a new enzyme-splitting lactose phosphate. Proc. Natl. Acad. Sci. U.S. **58**:274–279.
17. Horowitz, N. H. 1945. On the evolution of biochemical synthesis. Proc. Natl. Acad. Sci. U.S. **31**:153–157.
18. Horowitz, N. H. 1965. The evolution of biochemical synthesis—retrospect and prospect, p. 15–23. *In* V. Bryson and H. J. Vogel (ed.), Evolving genes and proteins. Academic Press, Inc., New York.
19. Hulley, S. B., S. B. Jorgensen, and E. C. C. Lin. 1963. Ribitol dehydrogenase in *Aerobacter aerogenes* 1033. Biochim. Biophys. Acta **67**: 219–225.
20. Ingram, V. M. 1963. The hemoglobins in genetics and evolution. Columbia Univ. Press, New York.
21. Jacob, F., and E. L. Wollman. 1961. Sexuality and the genetics of bacteria, p. 166–167. Academic Press, Inc., New York.
22. Kennedy, E. P., and G. A. Scarborough. 1967. Mechanism of hydrolysis of o-nitrophenyl-β-galactoside in *Staphylococcus aureus* and its significance for theories of sugar transport. Proc. Natl. Acad. Sci. U.S. **58**:225–228.
23. Kundig, W., S. Ghosh, and S. Roseman. 1964. Phosphate bound to histidine in a protein as an intermediate in a novel phospho-transferase system. Proc. Natl. Acad. Sci. U.S. **52**:1067–1074.
24. Leive, L., and B. D. Davis. 1965. The transport of diaminopimelate and cystine in *Escherichia coli*. J. Biol. Chem. **240**:4362–4369.
25. Lerner, S. A., T. T. Wu, and E. C. C. Lin. 1964. Evolution of catabolic pathway in bacteria. Science **146**:1313–1315.
26. Lester, G., and D. M. Bonner. 1957. Genetic control of raffinose utilization in *Escherichia coli*. J. Bacteriol. **73**:544–552.
27. Lewis, E. B. 1951. Pseudoallelism and gene evolution. Cold Spring Harbor Symp. Quant. Biol. **16**:159–174.
28. Lin, E. C. C. 1961. An inducible D-arabitol dehydrogenase from *Aerobacter aerogenes*. J. Biol. Chem. **236**:31–36.
29. Lin, E. C. C., S. A. Lerner, and S. E. Jorgensen. 1962. A method for isolating constitutive mutants for carbohydrate-catabolizing enzymes. Biochim. Biophys. Acta **60**:422–424.
30. Lin, E. C. C., A. P. Levin, and B. Magasanik. 1960. The effect of aerobic metabolism on the inducible glycerol dehydrogenase of *Aerobacter aerogenes*. J. Biol. Chem. **235**:1824–1829.
31. Lin, E. C. C., and B. Magasanik. 1960. The activation of glycerol dehydrogenase from *Aerobacter*

aerogenes by monovalent cations. J. Biol. Chem. **235**:1820–1823.

32. Magasanik, B., M. S. Brooke, and D. Karibian. 1953. Metabolic pathways of glycerol dissimilation. A comparative study of two strains of *Aerobacter aerogenes*. J. Bacteriol. **66**:611–619.
33. McPhedran, P., B. Sommer, and E. C. C. Lin. 1961. Control of ethanol dehydrogenase levels in *Aerobacter aerogenes*. J. Bacteriol. **81**:852–857.
34. Mortimer, D. C. 1952. Paper chromatographic separation of some biologically important phosphate esters. Can. J. Chem. **30**:653–660.
35. Mortlock, R. P., D. D. Fossitt, D. H. Petering, and W. A. Wood. 1965. Metabolism of pentoses and pentitols by *Aerobacter aerogenes*. III. Physical and immunological properties of pentitol dehydrogenases and pentulokinases. J. Bacteriol. **89**:129–135.
36. Mortlock, R. P., D. D. Fossitt, and W. A. Wood. 1965. A basis for utilization of unnatural pentoses and pentitols by *Aerobacter aerogenes*. Proc. Natl. Acad. Sci. U.S. **54**:572–579.
37. Mortlock, R. P., and W. A. Wood. 1964. Metabolism of pentoses and pentitols by *Aerobacter aerogenes*. I. Demonstration of pentose isomerase, pentulokinase, and pentitol dehydrogenase enzyme families. J. Bacteriol. **88**: 838–844.
38. Mortlock, R. P., and W. A. Wood. 1964. Metabolism of pentoses and pentitols by *Aerobacter aerogenes*. II. Mechanism of acquisition of kinase, isomerase, and dehydrogenase activity. J. Bacteriol. **88**:845–849.
39. Nordlie, R. C., and H. J. Fromm. 1959. Ribitol dehydrogenase. II. Studies on the reaction mechanism. J. Biol. Chem. **234**:2523–2531.
40. Novick, A., and M. Weiner. 1957. Enzyme induction as an all-or-none phenomenon. Proc. Natl. Acad. Sci. U.S. **43**:553–566.
41. Ornstein, L., and B. J. Davis. 1962. Disc electrophoresis. Distillation Products Ind., Rochester, N.Y., and Canal Industrial Corp., Bethesda, Md.
42. Prestidge, L. S., and A. B. Pardee. 1965. A second permease for methyl-thio-β-D-galactoside in *Escherichia coli*. Biochim. Biophys. Acta **100**: 591–593.
43. Rotman, B., A. K. Ganeson, and R. Guzman. 1968. Transport systems for galactose and galactosides in *Escherichia coli*. II. Substrate and inducer specificities. J. Mol. Biol., *in press*.
44. Rush, D., D. Karibian, M. L. Karnovsky, and B. Magasanik. 1957. Pathways of glycerol dissimilation in two strains of *Aerobacter aerogenes*: enzymatic and tracer studies. J. Biol. Chem. **226**:891–899.
45. Schaefler, S., and I. Schenkein. 1968. β-Glucoside permeases and phospho β-glucosidases in *Aerobacter aerogenes*: relationship with cryptic phospho β-glucosidases in Enterobacteriaceae. Proc. Natl. Acad. Sci. U.S. **59**:285–292.
46. Simoni, R. D., M. Levinthal, F. D. Kundig, W. Kundig, B. Anderson, P. E. Hartman, and S. Roseman. 1967. Genetic evidence for the role of a bacterial phosphotransferase system in sugar transport. Proc. Natl. Acad. Sci. U.S. **58**:1963–1970.
47. Tanaka, S., D. G. Fraenkel, and E. C. C. Lin. 1967. The enzymatic lesion of strain MM-6, a pleiotropic carbohydrate-negative mutant of *Escherichia coli*. Biochem. Biophys. Res. Commun. **27**:63–67.
48. Tanaka, S., S. A. Lerner, and E. C. C. Lin. 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93**: 642–648.
49. Tanaka, S., and E. C. C. Lin. 1967. Two classes of pleiotropic mutants of *Aerobacter aerogenes* lacking components of a phosphoenolpyruvate-dependent phosphotransferase system. Proc. Natl. Acad. Sci. U.S. **57**:913–919.
50. Winkler, H. H. 1966. A hexose-phosphate transport system in *Escherichia coli*. Biochem. Biophys. Acta. **117**:231–240.
51. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200–2211.
52. Wood, W. A., M. J. McDonough, and L. B. Jacobs. 1961. Ribitol and D-arabitol utilization by *Aerobacter aerogenes*. J. Biol. Chem. **236**: 2190–2195.